Isolation and identification of pathogenic bacteria associated with fish and fishery product- *vibrio Cholerae* and *vibrio parahaemolyticus*

Scope:

The Technical Specification describes a method for detection of *Vibrio parahaemolyticus* and *Vibrio cholerae* in products intended for human consumption or animal feeds and environmental samples from food production and food handling areas.

Principle:

The detection of *V. parahaemolyticus* and *V. cholerae* requires four successive stages. An enrichment medium, alkaline saline peptone water (ASPW), is inoculated with the test sample and incubated at 37°C for 6 h (±1 h) for deep frozen products, or 41.5°C for 18 h (±1 h). This is then used to inoculate the ASPW for a second enrichment stage. The medium is incubated at 41.5°C for 18 h (±1 h). The cultures obtained from both of the enrichment stages are used to inoculate thiosulphate citrate bile and sucrose (TCBS) agar and a second solid selective medium of choice. The TCBS plates are incubated at 37°C and examined after 24 h (±3 h). The medium of choice is incubated according to the manufacturer‟s instructions. Presumptive colonies are subcultured and confirmed using appropriate biochemical tests.

Equipment:

a) Autoclave

b) Incubator at 37ºC (±1°C)

c) Incubator or water bath at 41.5ºC (±1°C)

d) Water bath adjustable from 44°C to 47°C

e) Water bath at 37ºC (±1°C)

Media/Reagents:

a) ASPW

b) Thiosulphate citrate bile and sucrose agar (TCBS)

c) Selective agar of choice

d) Saline nutrient agar

e) Oxidase reagents

f) Saline triple sugar iron (TSI) agar

g) Saline medium for detection of ornithine decarboxylase

h) Saline medium for detection of lysine decarboxylase

i) Saline medium for detection of arginine dihydrolase

j) Reagent for detection of β-galactosidase

k) Saline medium for detection of indole

l) Saline peptone waters

m) Sodium chloride solution

Sampling:

Samples must be received in an intact food grade plastic bag and properly packed in a cool box with ice packs to reach a temperature of less than 8˚C within 4 hours and then maintain this for at least 24 hours. Such samples should not be received frozen.

Vibrio spp. can grow very rapidly in seafood at ambient temperature, and samples must be chilled to below 10ºC immediately and then analysed as quickly as possible. However, the cells are easily damaged by rapid cooling and samples should not be cooled by direct contact with ice.

Samples from harvesting areas should have been rinsed, but not immersed, and drained at time of sampling, and should be regarded as unsatisfactory if they are received in the laboratory with the sample container leaking, the shellfish covered in mud, or immersed in water or mud/sand. Choose shellfish that are alive and discard all dead shellfish and those with obvious signs of damage. Select the appropriate number of shellfish depending on the species. More shellfish can be used, if necessary, to produce the required volumes for each analysis. Samples should be examined immediately or stored at 3ºC (±2˚C) for no more than 24 hours until examination.

Sample preparation:

Sample preparation procedures for shellfish typically require pooling 10- 12 individual animals. The pooled sample is then homogenised using a sterile high-speed blender. If sample dilutions are required they should be prepared with a diluent containing salt, such as phosphate buffered saline (PBS).

Mud and sediment adhering to the shellfish should be removed prior to opening the shellfish by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be reimmersed in water as this may cause them to open. Open all selected shellfish as described

below with a flame-sterilised shucking knife, and empty meat and liquor into a beaker. To flame sterilise the shucking knife, place the knife in the beaker of ethanol and sterilise using an electric Bunsen system. Allow the knife to cool before using. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

Oysters and Clams - Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the beaker. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into a beaker.

Mussels and Cockles - Insert the knife between the shells of the animal and separate the shells with a twisting motion of the knife. Collect the liquor from the animal in the beaker, then cut the muscle between the shells and scrape the contents into a beaker.

Stomacher procedure: For homogenisation weigh 25g (±1g) of shellfish flesh and intra-valvular fluid into at least three stomacher bags, to avoid small pieces of shell from puncturing the bags. Remove excess air from the bag and operate the stomacher for 3 minutes at normal speed. Add sterile ASPW. Stomach at „normal" speed for a further 3 minutes.

Homogenisation: According to the sensitivity required, a portion of the sample is homogenized in ×9 the volume of ASPW. If there is a large quantity, the ASPW should be warmed to 37°C prior to inoculation with the test portion. If the dilution and incubation cannot be carried out on the same day, the initial suspension should be stored at 5ºC (±3°C) until the following day. Storage at refrigeration temperatures should be avoided or kept to a minimum as this will reduce levels of *V. parahaemolyticus* and *V. cholerae* in the sample. The initial suspension is incubated at 37°C for 6 h (±1 h) for deep-frozen products or 41.5°C for 6 h (±1 h) for fresh, dried, or salted products. Care should be taken to apply the whole method to products with a high salt content, as the final salt concentration in the medium might alter the characteristics.

Procedure:

For the second selective enrichment, 1 ml from the surface of the first selective enrichment culture is transferred to a tube containing 10 ml ASPW. The tube is incubated at 41.5°C for 18 h (±1 h).

The cultures obtained from both the first and second selective enrichments are streaked on to TCBS and the second isolation medium plates so that well separated colonies develop. The TCBS plates are incubated inverted at 37°C for 24 h (±3 h) and examined for typical colonies. *V. parahaemolyticus* colonies are smooth, green (sucrose negative) and 2–3 mm in

diameter. *V. cholerae* colonies are smooth, yellow (sucrose positive) and 2–3 mm in diameter. The plates of the second medium are incubated for the time/temperature recommended by the manufacturer, and also examined for typical colonies of *V. parahaemolyticus* or *V. cholerae.*

At least five typical colonies of each *Vibrio* species being tested for should be picked off selective agar plates and streaked on to plates of saline nutrient agar or used to inoculate slants of saline nutrient agar. The plates or slants are incubated at 37°C for 24 h (±3 h).

For confirmation, appropriate commercially available biochemical test kits may be used. The bacterial inoculums must always be prepared in a sufficiently saline medium or dilution fluid. Alternatively, colonies can be confirmed using the following tests. For a first screening for presumptive *V. parahaemolyticus* or *V. cholerae* cultures, the following tests are carried out.

The oxidase test should be carried out on the isolates. Gram stains and motility tests should also be carried out on the cultures. Any Gram-negative, motile cultures giving positive oxidase tests should be retained for further tests.

A slope of saline TSI agar is stabbed to the bottom of the agar butt and streaked longitudinally along the slope. It is incubated at 37°C for 24 h (±3 h). *V. parahaemolyticus* typically produces a red (alkaline) slant and a yellow (acid) butt, without the formation of or gas. *V. cholerae* typically produces a yellow (acid) slant and a yellow (acid) butt, without the formation of hydrogen sulphide or gas. The incubation time should not exceed 24 h as the yellow slant of *V. cholerae* may turn red after that time.

Saline ornithine decarboxylase medium is inoculated just below the surface, and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and decarboxylation of ornithine). A negative reaction is indicated by a yellow colour.

Saline lysine decarboxylase medium is inoculated just below the surface, and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and decarboxylation of lysine). A negative reaction is indicated by a yellow colour.

Saline arginine dihydrolase medium is inoculated just below the surface and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and dihydrolation of arginine). A negative reaction is indicated by a yellow colour.

To test for β -galactosidase activity, a colony is suspended in 0.25 ml saline solution. A drop of toluene is added and the tube is shaken. The tube is placed in a water bath at 37°C and allowed to stand for approximately 5 min. Then 0.25 ml β -galactosidase reagent is added

and the contents of the tube mixed. The tube is placed back in the water bath at 37°C and left for 24 h (±3 h), examining it from time to time. A yellow colour indicates the presence of β -galactosidase. Often, this reaction is visible after 20 min. An absence of colour after 24 h indicates a negative reaction. Commercially available paper disks may also be used.

To carry out the indole test, 5 ml tryptone-trytophan saline medium is inoculated with the suspect colony. It is incubated at 37°C for 24 h (±3 h). After incubation, 1 ml Kovac‟s reagent is added. The formation of a red ring indicates a positive reaction (formation of indole), whereas a yellowbrown ring signifies a negative reaction.

To test halotolerance a series of peptone waters with increasing salt concentration is prepared (0%, 2%, 4%, 6%, 8% and 10%). A suspension of a colony is prepared and each of the tubes is inoculated.

The tubes are incubated at 37°C for 24 h (±3 h). Growth is indicated by turbidity in the tube.

Typically, *V. parahaemolyticus* strains decarboxylate ornithine and lysine, give a negative reaction for arginine dihydrolase and β -galactosidase activity, and a positive indole reaction. *V. parahaemolyticus* requires salt for growth and will grow in up to 8% salt concentrations but not at 10%.

Typically, *V. cholerae* strains decarboxylate ornithine and lysine, give a negative reaction for arginine dihydrolase and positive reactions for β -galactosidase activity and production of indole. *V. cholerae* will grow in peptone water containing 0% and 2% salt, but will not grow at salt levels of 6% and higher.

Confirmation tests should be carried out on cultures that do not show growth in 10% saline peptone water and which give a negative arginine dihydrolase reaction.

Final confirmation and determination of pathogenicity factors are complicated, and best performed by sending cultures to a specialist/reference laboratory. Cultures should be sent on saline nutrient agar slopes.

Result and Comment: